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Running title: Host Strains Optimized for Polyene Glycosylation Engineering

Streptomyces nodosus Host Strains Optimized for Polyene Glycosylation
Engineering

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Abbreviations: ESMS, electrospray mass spectrometry; GT, glycosyltransferase;
LC-MS, liquid chromatography-mass spectrometry

The AmphDI glycosyltransferase transfers a mycosaminyl sugar residue from GDP onto 8-deoxyamphoteronolide B, the aglycone of the antifungal amphotericin B. In this study the *amphDI* gene was inactivated in *Streptomyces nodosus* strains lacking the AmphN cytochrome P450. The new mutants produced 8-deoxy-16-methyl-16-descarboxyl amphoteronolides in high yield. These strains and aglycones should prove valuable for *in vivo* and *in vitro* glycosylation engineering.

Key words: Polyene macrolide biosynthesis; glycosylation engineering; amphotericin

There is considerable interest in modifying the structures of bioactive natural products to improve pharmacological properties. Manipulation of biosynthetic genes in producer organisms can lead to the production of analogs at low cost by high yielding fermentation processes.^{1, 2)} This approach is being applied to polyene macrolides, antifungal antibiotics that are also active against pathogenic prion proteins, *Leishmania* parasites, and enveloped viruses.³⁾

One of the most important of these polyenes is amphotericin B (**1**), produced by *Streptomyces nodosus* (Fig. 1). Biosynthesis begins with formation of the macrolactone core, 8-deoxy-16-descarboxyl-16-methyl-amphoteronolide B (**2**). This is assembled by a modular polyketide synthase that also forms the 28-29-dihydro analog **3** (8-deoxy-16-descarboxyl-16-methyl-amphoteronolide A) as a co-metabolite.⁴⁾ **2** and **3** undergo the same three late modifications to form the final products amphotericins B (**1**) and A. AmphN cytochrome P450 oxidizes the C-16 methyl branch to a carboxyl group, the AmphDI glycosyl transferase (GT) catalyzes transfer of a mycosamine (3-amino-3,6-dideoxy-D-mannose) residue to C-19, and the AmphL P450 catalyzes hydroxylation at C-8.⁵⁾

Deletion of the *amphN* gene yields less toxic analogs in which the carboxyl group at C-16 is replaced with a methyl group.^{6, 7)} In this study, we took a step towards further improvement by glycosylation engineering, a developing

technology that is being applied to other natural products. Altering sugar residues has enhanced the properties of doxorubicin, glycopeptide antibiotics, and several other bioactive compounds.⁸⁾

Mycosamine is synthesized from GDP-D-mannose *via* GDP-4,6-dideoxy-4-oxo-D-mannose and GDP-3,6-dideoxy-3-oxo-D-mannose intermediates. The final step is a transamination catalyzed in *S. nodosus* by AmphDII mycosamine synthase. Inactivation of the *amphDII* gene led to the formation of amphoteronolides modified with a neutral deoxyhexose. This sugar is thought to be D-rhamnose (6-deoxy-D-mannose), formed as a shunt product by reduction of one of the oxo-sugar intermediates in mycosamine biosynthesis. The enzyme that catalyzes this reduction has not been identified. Further re-design of amphotericin glycosylation has been achieved using genes from *Streptomyces aminophilus*. This organism produces perimycin, an aromatic heptaene that is glycosylated with perosamine (4-amino-4,6-dideoxy-D-mannose) rather than mycosamine.⁹⁾ Perosamine is synthesized by transamination of GDP-4,6-dideoxy-4-oxo-D-mannose. Introduction of a *perDII* perosamine synthase gene into a *S. nodosus amphDII* mutant resulted in the production of small amounts of 19-(*O*)-perosaminyl-amphoteronolide B along with more abundant deoxyhexosylated amphoteronolides and aglycones as major polyene products. The yield of the perosaminyl analog was increased by inclusion of a hybrid GT named Hap2. This consisted of the N-terminal aglycone-binding domain of the AmphDI mycosaminyltransferase and the C-terminal GDP-perosamine binding domain of the *S. aminophilus* PerDI perosaminyltransferase.⁹⁾ The hemolytic and antifungal activities of perosaminyl-amphoteronolide B were similar to those of amphotericin B, but this work helped to define the boundary between the sugar donor-binding and aglycone acceptor-binding domains in polyene glycosyltransferases.

Further re-design of AmphDI might eventually make possible glycosylation of amphoteronolides and related polyene aglycones with the dTDP-glucose- and UDP-glucose-derived deoxysugars that appear in other glycosylated natural products. The range of pyrimidine nucleotide-linked deoxysugars is greater than

that biosynthesized from GDP-mannose. After attachment to aglycones, some dTDP-glucose derived sugars are further modified with one or more additional sugars by iterative or extending GTs.¹⁰⁾ It may eventually be possible to use biosynthetic engineering to add longer oligosaccharide chains to polyenes. Chemical modification studies have shown that this increases water-solubility and reduces toxicity.^{11,12)}

Alteration of GT substrate specificity requires replacement of key amino acid residues or domain swapping.^{13,14)} The task is challenging and requires systems for testing large numbers of recombinant transferases for novel activities. For polyene GTs, a rapid and sensitive assay might be based on the fact that aglycones are not antifungal, whereas glycosylation confers activity. Detection of antibiotic activity is amenable to high-throughput screening. In contrast, product isolation and characterization is time consuming.

Targeted inactivation of the *amphDI* gene should yield host strains that can be used to identify engineered GTs that convert inactive amphoteronolides to active glycosylated forms. Here we carried out this disruption and found that the mutation can be complemented by plasmid-encoded GTs. Gene replacement was carried out with strains *amphNM* and *amphDII-NM* which produce 16-descarboxyl-16-methyl amphoteronolides modified with mycosamine and deoxyhexose respectively.

Primers D1F and D1-inr [5' GTACGAATTCAGGCCTGCTCATCGGCTTGAAGAACACC 3' and 5' GAGAGCATGCGCTGATTCGGGTACGAGCTGGCGATCACCAAG 3'] were used to amplify the 5' region of the *amphDI* gene and upstream DNA (nucleotides 63402 to 65321 of sequence accession no. AF357202). Primers D1-inf and D1R [5' GATCGCATGCTCTCGATCACCATCAGGGCGG 3' and 5' AGCTAAGCTTAGATCTGCCACTGATGCCGAAGGAGGAG 3'] were used to amplify the 3' end of *amphDI* and downstream DNA (nucleotides 65333 to 67343 of AF357202). (In the primer sequences, *EcoR* I and *Hind* III sites are italicized, *Sph* I sites are in bold, and the *Stu* I and *Bgl* II sites are underlined). The two PCR

products were ligated through *Sph* I sites incorporated into the D1-inr and D1-inf primers. The ligated DNA was digested with *Eco*R I and *Hind* III and cloned into pUC118. The resulting construct, pUC118- Δ *amphDI*, contained a mutated *amphDI* gene in which the wild-type sequence AGC-ATG-TGC-CAG (encoding Ser¹²⁸-Met¹²⁹-Cys¹³⁰-Gln¹³¹) was replaced with AGC-ATG-CGC-TGA, which ends with an in-frame stop codon and contains an *Sph* I restriction site. The insert was excised with *Bgl* II and *Stu* I and cloned between the *Bam*H I and *Sca* I sites of phage KC-UCD1 to give KC- Δ *amphDI*. The recombinant phage was propagated on *S. nodosus amphNM* and *S. nodosus amphDII-NM*.^{6,9)} Gene replacement was carried out as described previously.¹⁵⁾ The required mutants were identified by amplifying the *amphDI* gene and testing for the presence of an *Sph* I site in the PCR product (Fig. 2). The strains were named *S. nodosus amphDI-NM* and *amphDI-DII-NM* respectively.

Polyenes were extracted and analyzed as described previously by ESMS or LC-MS.¹⁶⁾ ESMS was performed on a triple quadrupole Micromass Quattro LC machine. For LC-MS, quadropole time-of-flight mass spectrometry was run on a XEVO instrument with Waters Acquity UP-LC (Waters Corporation, Micromass UK Ltd., Manchester, UK). The polyenes discussed have been partially characterized in previous work.^{6,9,16)} The aim here was to carry out sufficient analysis to identify the products made by the new mutant strains and transformants.

ESMS analysis of crude extracts of both the DI-NM and the DI-DII-NM mutants revealed polyenes **2** and **3** ($[M - H]^- = 731.5$ and 733.5 respectively; $[M + Na]^+ = 755.5$ and 757.5). Earlier studies gave unstable lysogens of *S. nodosus amphNM* that occasionally produced **2** and **3** rather than glycosylated forms, presumably as a result of unpredictable spontaneous deletions in glycosylation genes.^{9,16)} This previous work made possible chemical characterization of **2**, but did not give a reliable producer strain for this compound. With both of the new mutants, **2** and **3** were consistently produced in high yields (about 50 mg each per liter) and both aglycones should prove valuable for chemical modification and for

in vitro glycosylation. The two aglycones can be separated efficiently because heptaene **2** is considerably less soluble in methanol than tetraene **3**.¹⁶⁾

The new strains were assessed as hosts for plasmid-encoded GTs. To construct an *amphDI* expression plasmid, the gene was amplified with primers AmphDI-F [5' AAAAAAGATCTCATATGGCGGCGGCGTCGGCGAATGCGATC 3'] and AmphDI-R [5' AAAAAGCTTGACTTCCTCGGTCAGTCGTT 3']. The product was digested with *Bgl* II and *Hind* III and cloned between the *Bam*H I and *Hind* III sites of expression vector pIAGO. The resulting plasmid construct, pIAGO-*amphDI*, was introduced into strains DI- NM and DI-DII-NM by protoplast transformation.

Transformation with empty pIAGO vector had no effect on polyene production by DI-DII-NM (Fig. 3, panel A). In strain DI-DII-NM, the plasmid-encoded AmphDI catalyzed efficient conversion of **2** to the deoxyhexosylated form **4** ($[M + Na]^+ = 917.5$) (Fig. 3, panel B). Aglycone **3** was also deoxyhexosylated to give **5** ($[M + Na]^+ = 903.5$).

The pIAGO-Hap2 plasmid contains genes for a hybrid AmphDI-PerDI GT and PerDII perosamine synthase. *S. nodosus* DI-DII-NM was transformed with this plasmid and polyenes were extracted. Analysis by ESMS revealed polyenes with masses appropriate for perosaminylated analogs **6** ($[M - H_2O + H]^+ = 876.5$; $[M + Na]^+ = 916.5$) and **7** ($[M - H_2O + H]^+ = 862.5$; $[M + Na]^+ = 880.5$), along with aglycones **2** and **3** and deoxyhexosylated forms **4** and **5**. LC-MS analysis revealed that Hap2 catalyzed the formation of perosaminylated and deoxyhexosylated macrolactones in approximately equal quantities (Fig. 3, panel C). The perosaminylated, deoxyhexosylated, and unglycosylated forms made up approximately 25%, 25%, and 50% of the total polyene respectively. Although the concentrations of NDP-sugars in *S. nodosus* are not known, these results suggest that the final product profile is influenced by GDP-sugar donor availability as well as by GT specificity.

Previously, the pIAGO-Hap2 plasmid was tested in *amphDII* mutants that retained active chromosomal *amphDI* genes, so that both Hap2 and AmphDI enzymes contributed to the formation of glycosylated polyene products. The new *amphDI* mutants make possible the assessment of individual plasmid-encoded GTs.

The *S. nodosus amphDI-NM* mutant retained the chromosomal *amphDII* gene for mycosamine synthase. Transformation of this strain with pIAGO-*amphDI* resulted in the production of mycosaminylated forms of **2** ($[M + H]^+ = 894.5$; $[M + Na]^+ = 916.5$) and **3** ($[M + H]^+ = 880.5$; $[M + Na]^+ = 902.5$). This indicates that the *amphDII* gene is expressed despite the nonsense mutation in *amphDI*. In some cases, such mutations prevent the expression of downstream genes within a transcription unit.¹⁷⁾

Crude extracts containing perosaminylated or mycosaminylated amphoteronolides were active against *Saccharomyces cerevisiae* in qualitative bioassays. Extracts containing less active deoxyhexosylated analogs were inhibitory after 10-fold concentration. The highest achievable concentrations of aglycones **2** and **3** had no detectable activity. These results indicate that screening for antifungal activity is sensitive enough to reveal *in vivo* glycosylation of amphoteronolides, at least with amino sugars.

Croatt and Carreira (2011) recently synthesized an amphotericin B analog in which mycosamine is replaced with 3-amino-3, 6-dideoxy-D-glucose.¹⁸⁾ This new analog retained antifungal activity. dTDP-3-amino-3, 6-dideoxy-D-aminoglucose is the penultimate intermediate in the formation of dTDP-mycaminose, which has been biosynthesized in heterologous streptomycete hosts.¹⁹⁾ Mycaminose is of particular interest because, after attachment to tylactone, it is modified with an L-mycarosyl sugar residue by an extending GT in the tylosin producer *Streptomyces fradiae*. Biosynthetic gene cassettes have also been constructed for dTDP-4-amino-4, 6-dideoxy-D-glucose.²⁰⁾ The *S. nodosus* genome already contains genes for the formation of dTDP-4, 6-dideoxy-4-oxo-glucose.²¹⁾ Only one or two additional genes are required for the formation of dTDP-aminodeoxyglucoses.

Thus the tools are now available for the identification of recombinant GTs that utilize dTDP-linked sugars to glycosylate polyene aglycones.

There has been significant progress in the characterization of the PimD cytochrome P450 that oxidizes the polyol chain of the tetraene pimaricin.²²⁾ Aglycones **2** and **3** are natural substrates for AmphN cytochrome P450, which oxidizes the C-16 methyl branch to form the exocyclic carboxyl group. The new strains producing these compounds should also assist *in vitro* and *in vivo* analyses of the mechanism of the AmphN-catalysed cytochrome P450 reaction.

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Figure legends

Fig. 1. Structures of Polyenes Described in This Work.

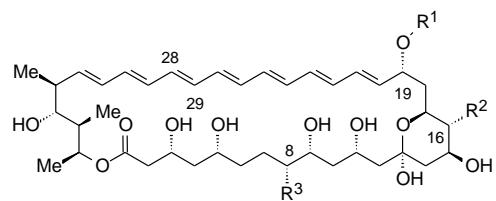
1, Amphotericin B; **2**, 8-deoxy-16-descarboxyl-16-methyl-amphoteronolide B; **3**, 28,29-dihydro **2**; **4**, 16-descarboxyl-16-methyl-19-*O*-deoxyhexosyl-amphoteronolide B; **5**, 8-deoxy-16-descarboxyl-16-methyl-19-*O*-deoxyhexosyl-amphoteronolide A; **6**, 16-descarboxyl-16-methyl-19-*O*-perosaminyl-amphoteronolide B; **7**, 8-deoxy-16-descarboxyl-16-methyl-19-*O*- perosaminyl-amphoteronolide A. **1**, **2**, **4**, and **6** are heptaenes and **3**, **5**, and **7** are tetraenes. 16-Descarboxyl-16-methyl-tetraenes do not undergo C-8 hydroxylation after glycosylation.

Fig. 2. Analysis of the *amphDI* –*DII-NM* Mutant by PCR.

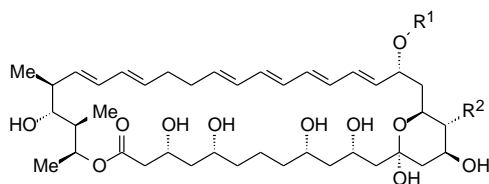
The gel shows *amphDI* region DNA amplified from *S. nodosus amphDI-DII-NM* (lanes 1 and 3) and from parent strain *S. nodosus amphDII-NM* (lanes 2 and 4). Samples analyzed on lanes 1 and 2 were untreated controls, those in lanes 3 and 4 were treated with *Sph* I. The primers used were AmphDI-F and AmphDI-R.

Fig. 3. Analysis of Polyenes from *S. nodosus* DI-DII-NM Transformed with pIAGO (panel A), pIAGO-*amphDI* (panel B), and pIAGO-Hap2 (panel C).

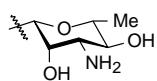
In the LC-MS analyses, heptaenes were detected by monitoring the A₄₀₅ of the column eluent. The relevant sections of the various chromatograms are shown. The separation of each peak into a doublet is an artifact. Heptaenes **2**, **4**, and **6** were identified by their molecular masses.



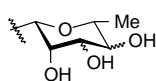
	R ¹	R ²	R ³	M _r
1	Mycosamine	COOH	OH	923.5
2	H	CH ₃	H	732.5
4	Deoxyhexose	CH ₃	OH	894.5
6	Perosamine	CH ₃	OH	893.5



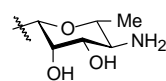
	R ¹	R ²	M _r
3	H	CH ₃	734.5
5	Deoxyhexose	CH ₃	880.5
7	Perosamine	CH ₃	879.5



Mycosamine



Deoxyhexose



Perosamine

Fig. 1 Stephens

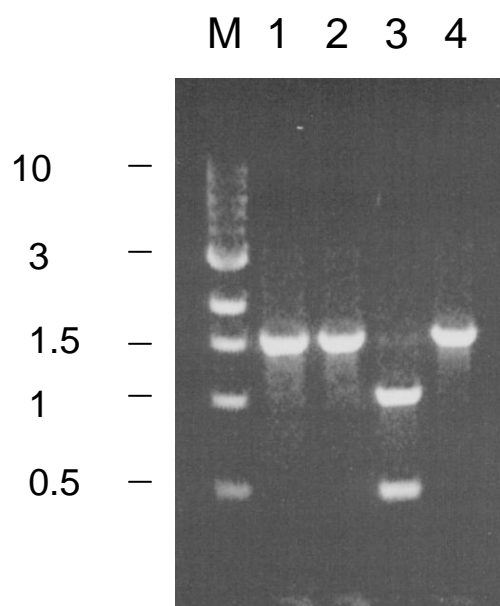


Fig. 2 Stephens

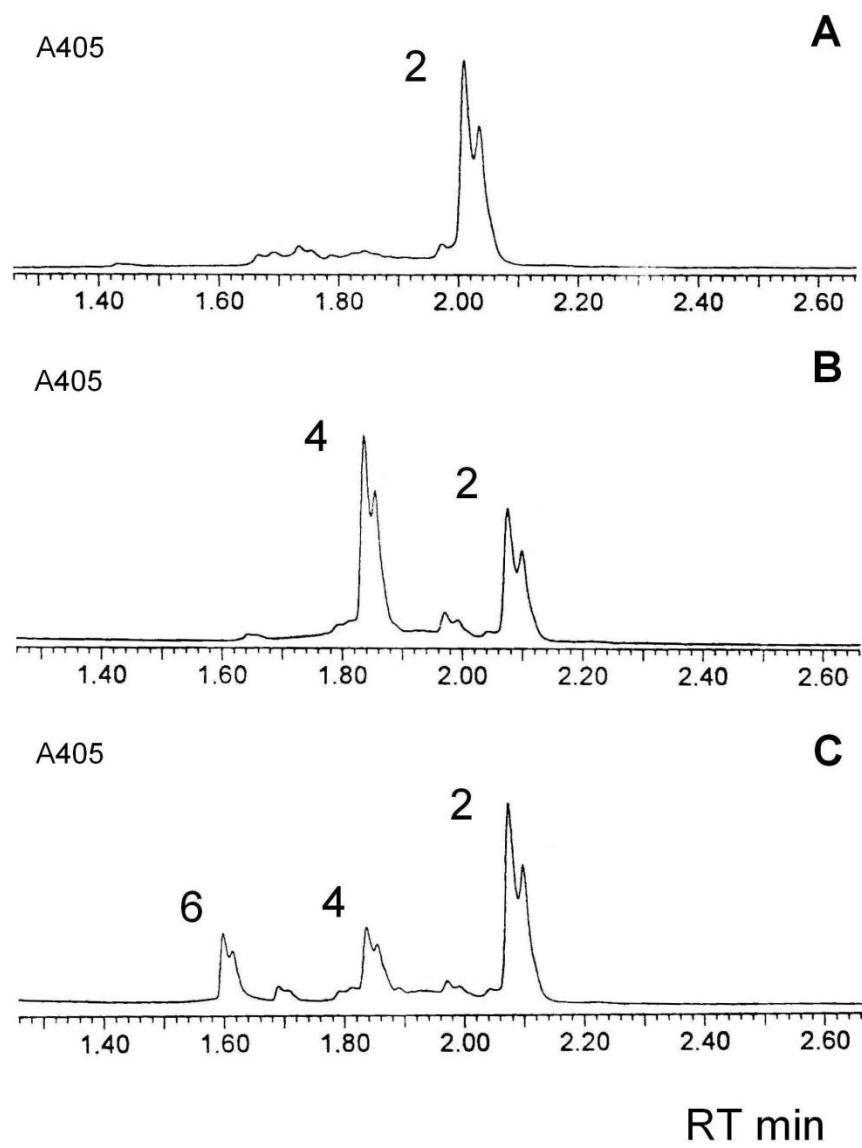


Fig. 3 Stephens